REMARKS

Interview Summary

Pursuant to 37 CFR §1.133(b), Applicants acknowledge with appreciation the in-person interview with the Examiner on December 8, 2005 during which the outstanding rejections of the claims were discussed.

Pending Claims

Claims 27-35, 38, 39, 42-46 and 49-51 are pending. Claims 31, 32 and 34 have been amended. Claims 49 and 50 has been canceled without prejudice. New claim 52 has been added. Accordingly, claims 27-35, 38, 39, 42-46, and 51-52 will be pending upon entry of the present amendment.

Claims 31 and 34 have been amended without prejudice to delete reference to conservative sequence modifications.

Claims 32 and 34 have been amended to specify that the conjugate comprises a melanoma antigen. Support for this amendment can be found throughout the specification as originally filed, for example, page 5, lines 3-9.

New claim 52 specifies that the molecular conjugate of claim 30 is produced as a recombinant fusion protein or a chemical conjugate. Support for new claim 52 can be found throughout the specification as originally filed, *e.g.*, page 35, lines 19-36.

The foregoing amendments should in no way be viewed as acquiescence to any rejection. Applicants reserve the right to pursue the claims as originally filed in this or subsequent applications. No new matter has been added.

Rejection of Claims 31, 34, 38 and 39 Under 35 U.S.C. §112, First Paragraph

Claims 31, 34, 38 and 39 are rejected as not being enabled. The Examiner states that:

> [g]iven the established unpredictability of the art, the instant specification would require a significant teaching to be enabled. In particular, it is unlikely that the generic modified conjugates encompassed by the claims would encompass conjugates modified in the CDR binding regions of the antibody portions of the conjugates. It is well-established that even single substitutions in the CDR regions of an antibody can have a dramatic, and unpredictable, effect on antibody binding (and, thus, function). See, for example, Kobayashi et al. (1999) wherein it is taught that even single conserved substitutions can have a large effect on antibody binding (see Figure 4; note the log scale). Note the breadth of the claims; the conjugates of the claims are not limited in

> the number of modifications/substitutions allowed. Thus, even antibodies in which all of the amino acids are changed would be encompassed by the claims.

Applicants respectfully traverse this rejection. However, to expedite prosecution, independent claims 31 and 34 have been amended without prejudice to delete reference to conservative sequence modifications. Therefore, this rejection should be moot.

Rejection of Claims 31, 34, 38 and 39 Under 35 U.S.C. §112, First Paragraph

Claims 31, 34, 38 and 39 are rejected as containing "insufficient written description to show that Applicant was in possession of the 'conservative sequence modifications' of the molecular conjugates of claims 31 and 34." The Examiner states that:

> [t]he specification neither defines the term nor discloses any of the modified conjugates of the claims. Given the essentially unlimited number of compositions encompassed by the claims, one of skill in the art would conclude that the specification fails to disclose a representative number of species to describe the clamed genus.

Applicants respectfully traverse this rejection. However, to expedite prosecution, independent claims 31 and 34 have been amended without prejudice to delete reference to conservative sequence modifications. Therefore, this rejection should be moot.

Rejection of Claims 27-30, 32, 33, 35, 38, 39, 50 and 51 Under 35 U.S.C. §103(a)

Claims 27-30, 32, 33, 35, 38, 39, 50 and 51 are rejected as being unpatentable over U.S. Patent No. 5,922,845 in view of Tuting *et al.* (1998) and Sallusto *et al.* (1995). The Examiner asserts that:

> [t]he '845 patent teaches a molecular conjugate comprising an antibody that binds to dendritic cells (DCs) (FcαR) and an antigen, wherein said antigen comprises a component of a pathogen or a tumor (cancer) antigen (see column 3, lines 49-59) . . . The reference further teaches that the molecular conjugates of the reference can be used to "harness the capabilities of white blood cells," *e.g.*, phagocytosis, for "enhancing the attack of these cells against cancer cells, cells of infectious microorganisms, and cells infected with pathogens."

The Examiner further states that "[t]he reference teaching differs from the claimed invention only in that it does not teach a molecular conjugate comprising an antibody that

binds to a human macrophage mannose receptor and the Pmel-17 tumor antigen." The Examiner relies on Sallusto *et al.* for teaching "that the human mannose macrophage receptor (which would be encoded by SEQ ID NO:7) can be employed for the uptake of antigen by DCs for presentation of said antigen to T cells." Thus, the Examiner concludes that it would have been obvious to produce a molecular conjugate "comprising an antibody that binds to white blood cells (which would include DCs) and tumor antigen, as taught by the '845 patent, employing an antibody that binds the human macrophage mannose receptor and Pmel-17 as the antigen."

With regard to motivation, the Examiner asserts that:

> one would have been motivated to employ an antibody that binds the human macrophage mannose receptor because it is more APC-specific than is the FcαR of the '845 patent, thus allowing for more efficient antigen uptake by PCs and more efficient antigen presentation to T cells. One of ordinary skill in the art at the time the invention was made would have been motivated to employ any of the well known tumor antigens in an anti-cancer therapeutic agent, such as Pmel-17 as taught by Tuting *et al.*, because of their availability and previous characteristics.

Applicants respectfully traverse this rejection. First, Applicants note that claim 35 is drawn to a molecular conjugate encoded by a particular nucleotide sequence which clearly would not have been obvious over the prior art given that human antibodies against the human mannose receptor had not even been generated at the time of the present invention. Accordingly, Applicants respectfully request that this rejection be withdrawn as it pertains to claim 35.

Claims 27-30, 32, 33, 35, 38, 39, 50 and 51 are drawn to molecular conjugates comprising a human monoclonal antibody that binds to the human macrophage mannose receptor, linked to an antigen. Thus, the relevant inquiry under 35 U.S.C. §103 for the invention as presently claimed is whether or not one of ordinary skill in the art at the time the application was filed would have been motivated to have linked a human anti-dendritic cell antibody to an antigen. For at least the following reasons, such motivation did not exist at the time of filing.

As was known in the art at the time of filing, dendritic cells are specialized cells of the immune system. Dendritic cells are the principle antigen presenting cells involved in primary immune responses. Their major function is to obtain antigen in tissues, migrate to lymphoid organs and activate T cells. Dendritic cells are capable of evolving from immature, antigen-

capturing cells to mature, antigen-presenting, T cell-priming cells; converting antigens into immunogens and expressing molecules such as cytokines, chemokines, costimulatory molecules and proteases to initiate an immune response.

As was also known in the art and as confirmed by Sallusto *et al.* (cited by the Examiner), immature monocyte-derived dendritic cells can efficiently capture and internalize mannosylated antigens and selected other carbohydrate containing ligands via the mannose receptor. To accomplish this task, the authors cite the following two critical characteristics of the mannose receptor to function in antigen presentation: "broad ligand specificity and the capacity to release ligands at low pH". Specifically, the authors focus on the fact that carbohydrate (*e.g.*, mannosylated antigen) interactions with the carbohydrate binding regions of the mannose receptor are low affinity interactions that are sufficiently strong enough to mediate selective binding, but weak enough to allow efficient dissociation of the ligands and antigens in the intracellular compartments.

In contrast, monoclonal antibodies were known in the art to bind target epitopes (e.g., target receptors) with high affinity (e.g., affinities far greater than the carbohydrate interactions between mannosylated antigens and the mannose receptor). This level of binding would not have been thought suitable for targeting antigens to the mannose receptor since it would have been expected that the antibody – antigen conjugate would fail to dissociate from the receptor once internalized. Indeed, antibodies which bind the human mannose receptor would not have been expected to exhibit the specialized multivalent properties of mannose receptor ligands necessary for antigen presentation, *i.e*, such antibodies would not have been expected to exhibit low affinity interactions capable of efficient dissociation of the ligands and antigens in the intracellular compartments. Therefore, it would not have been obvious in the least that antibodies directed to the mannose receptor could be used to target antigens to dendritic cells for efficient antigen uptake and presentation, or for any other purpose whatsoever.

Moreover, Sallusto *et al.* make a clear distinction between mannose receptor mediated-endocytosis of ligands and the internalization of Fc receptors and their ligands; the latter of which results in delivery to lysozomes and the degradation of both the ligand and the receptor. Accordingly, one of ordinary skill would not have been motivated to have used antibodies to target antigens to dendritic cells since such antibodies would have been

expected also to bind the Fc receptors expressed on these cells, thus, resulting in degradation of the ligand and the receptor.

The second mechanism of antigen uptake is macropinocytosis. Specifically, macropinocytosis in dendritic cells allows continuous capture of macrosolutes present in the fluid phase. The macrosolutes that are taken up in the fluid phase accumulate with time in the endocytic compartment, where they are loaded on newly synthesized and recycling MHC class II molecules but may also be released into the cytosol, where they become accessible to the class I antigen presentation pathway.

Again, such mechanisms for capturing antigens are far different from antibody-mediated antigen presentation, and would not have been thought suitable for antibody-based vaccines. Indeed, prior to the present invention, the mannose receptor was not recognized to capture and process antigens via antibodies. In fact, the anti-mannose receptor antibodies developed by Applicants as part of the present invention were initially selected by Applicants based on their superior functional properties (e.g., efficient internalization and antigen presentation) before it was known what specific receptor on dendritic cells the antibodies bound to. It was entirely unexpected when the antibodies were later characterized as binding to the human mannose receptor. Moreover, the antibody-mediated internalization observed by Applicants using the presently claimed anti-mannose receptor antibodies was found to involve mechanisms which were entirely different and independent of those involved in model mannose receptor ligand internalization (see, V. Ramakrishna *et al.* (2004) J. Immunol. 2846-2852; enclosed as Appendix A).

Accordingly, because at the time of the present invention it was understood that antigens were presented via the mannose receptor by mechanisms which differed from antibody-targeted mechanisms and would not have been thought suitable for developing antibody-based vaccines, there would not have been sufficient motivation to have made the presently claimed molecular conjugates comprising a human monoclonal antibody that binds to the human macrophage mannose receptor linked to an antigen. Importantly, it was not until Applicants' invention that antibodies to the human macrophage mannose receptor were unexpectedly discovered to be useful for antigen presentation. Therefore, the presently claimed invention would not have been obvious and Applicants respectfully request withdrawal of the present rejection.

Rejection of Claims 27-30, 32, 33, 35, 38, 39, 50 and 51

Under 35 U.S.C. §112, First Paragraph

Claims 27-30, 33, 35, 38, 39, 50 and 51 are rejected as containing new matter. The Examiner states that the specification and the claims as originally filed do not provide support for a human monoclonal antibody that binds to the human macrophages mannose receptor as encompassed by the claims. According to the Examiner, "the specification discloses only the B11 antibody [comprising] SEQ ID NOs:2 and 4 and not the generic antibody of the claims."

Applicants respectfully traverse this rejection and note that the specification explicitly describes a genus of human antibodies that bind to the human MMR. For example, original claim 6 (as filed with the application on November 7, 2001) was drawn to an isolated human monoclonal antibody that binds to the "human macrophage mannose receptor" (MMR). Molecular conjugates encompassing such antibodies are also taught, *e.g.*, at pages 4, lines 28-35, of the application as originally filed which teaches that the antibodies (*e.g.*, the antibodies of original claim 6) can be functionally linked to one or more antigens to form a vaccine conjugate.

Additional support for the anti-MMR antibodies currently claimed can be found, for example, at page 3, lines 7-16, which states that:

> [i]n another embodiment, human antibodies of the present invention are characterized by specific binding to human dendritic cells and one or more of the following properties:
>
> a) the ability to bind to the mannose receptor present on human dendritic cells with a binding equilibrium association constant (Ka) of at least about 10^7 M^{-1};
>
> b) the ability to opsonize human dendritic cells;
>
> c) the ability to be internalized after binding to human dendritic cells; and
>
> d) the ability to block binding to the mannose receptor on human dendritic cells.

A specific example of the claimed anti-MMR antibodies that meets the above-recited criteria is the B11 antibody which is characterized, for example, in Examples 2-4 (pages 56-67); see, also, page 2, lines 20-29, which describes that:

> [i]n other embodiments, the human antibodies are characterized by binding to particular novel epitopes (e.g., receptors) on dendritic cells. For example, specific antibodies of the present invention include human monoclonal antibody B11 comprising the heavy and light chain amino acid sequences shown in SEQ ID NOS: 2 and 4, respectively, which binds to the human macrophage mannose receptor (also referred to herein as "human B11 antigen") . . .

Accordingly, contrary to the Examiner's assertions, the present specification contains clear and explicit support for a genus of antibodies which bind human MMR as currently claimed. Thus, one of ordinary skill would clearly recognize that Applicants had possession of the claimed invention at the time of filing based on Applicants' written description, as well as the particular embodiments (species) of the claimed invention exemplified by Applicants such as the B11 antibody. Therefore, the subject matter of claims 27-30, 33, 35, 38, 39, 50 and 51 does not constitute new matter and Applicants respectfully request that the rejection be withdrawn.

SUMMARY

In view of the foregoing amendments and arguments, reconsideration and withdrawal of all the rejections and allowance of this application with all pending claims are respectfully requested. If a telephone conversation with Applicants' Attorney would expedite the prosecution of the above-identified application, the Examiner is urged to call (617) 227-7400.

Applicants believe no additional fee is due with this response. However, if an additional fee is due, please charge our Deposit Account No. 12-0080, under Order No. CDJ-166CPRCE from which the undersigned is authorized to draw.

Dated: March 30, 2006

Respectfully submitted,

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The Journal of Immunology

Mannose Receptor Targeting of Tumor Antigen pmel17 to Human Dendritic Cells Directs Anti-Melanoma T Cell Responses via Multiple HLA Molecules

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Targeting recycling endocytic receptors with specific Abs provides a means for introducing a variety of tumor-associated Ags into human dendritic cells (DCs), culminating in their efficient presentation to T cells. We have generated a human mAb (B11) against the mannose receptor that is rapidly internalized by DCs through receptor-mediated endocytosis. By genetically linking the melanoma Ag, pmel17, to Ab B11, we obtained the fully human fusion protein, B11-pmel17. Treatment of DCs with B11-pmel17 resulted in the presentation of pmel17 in the context of HLA class I and class II molecules. Thus, potent pmel17-specific T cells were cytotoxic toward gp100$^+$ HLA-matched melanoma targets, but not HLA-mismatched melanoma or gp100$^-$ nonmelanoma tumor lines. Importantly, competitive inhibition of lysis of an otherwise susceptible melanoma cell line by cold targets pulsed with known gp100 CD8 T cell epitopes as well as a dose-dependent proliferative response to Th epitopes demonstrates that DCs can process targeted Ag for activation of cytotoxic as well as helper arms of the immune response. Thus, the specific targeting of soluble exogenous tumor Ag to the DC mannose receptor directly contributes to the generation of multiple HLA-restricted Ag-specific T cell responses. ***The Journal of Immunology,*** **2004, 172: 2845–2852.**

Professional APCs (pAPCs),[4] such as dendritic cells (DCs) and macrophages (Mϕ), play an important role in regulating the immune response as a first line of defense by responding to danger signals and engaging the cellular arm of the immune repertoire (1–3). DCs are specialized APCs, in that they can be further manipulated to induce potent Th1 immunity by stimulating both CD4$^+$ and CD8$^+$ T cell responses (4–6) or promote a tolerogenic response where control of autoimmunity is desired (7). Although T cell responses to peptides presented by DCs have been adequately demonstrated both in vitro as well as in DC-based peptide vaccines, such responses have been difficult to obtain with soluble proteins due to their poor uptake and inefficient delivery to MHC class I processing compartments (8).

One means to facilitate the uptake and processing of exogenous soluble Ag has been to deliver Ag via immune complexes directed to MHC class II and FcγRIs present on APCs (9–14). Alternatively, Ags can be targeted to members of the C-type lectin family of endocytic receptors expressed on DCs, such as DC-SIGN (15), DEC-205 (16, 17), CD1 (18), LOX-1 (19), and the mannose receptor (MR) (20–24). Although the FcR, MHC class II, and DC-SIGN have been successfully targeted with model Ags, the path of Ag presentation in these murine models appears to be predominantly MHC class II restricted. In the case of MR targeting with mannosylated MUC1 Ag, both MHC class I- and class II-mediated presentation in humans was reported (25, 26). In the present study we have developed a fully human anti-mannose receptor Ab (B11) as a vehicle to deliver the human tumor Ag pmel17 directly to DCs. We demonstrate that monocyte-derived DCs previously loaded with the B11-pmel17 fusion protein are sufficient to direct the cellular component of the immune response, consisting of a potent HLA class I-dependent, CD8$^+$ CTL response in addition to a HLA class II-dependent, proliferative CD4$^+$ Th response.

Materials and Methods

Cells and reagents

Human PBMCs were isolated from normal donor heparinized Leukopaks (Biological Specialty Corp., Colmar, PA) using a standard density gradient centrifugation method (Lymphocyte Separation Medium; ICN Biomedical, Irvine, CA). HLA information was obtained from available records. Two donor samples described in this study were HLA-A2, 31 B13, 35, DR2 (donor 1) and HLA-A1, 30 B7, 13, DR7 (donor 2). All materials were handled aseptically according to standard guidelines. Melanoma cell lines and Abs to HLA (W6/32, anti-pan class I; BB7.2, anti-HLA-A2; B1.2.3, anti-B and -C locus; ME1.2, anti-B7/27; L243, anti-pan DR; and isotype-matched control Abs) were obtained from American Type Culture Collection (Manassas, VA). T cell stimulatory anti-CD3 Ab was obtained from BD Biosciences (San Jose, CA). Unless otherwise indicated, all cytokines were purchased from PeproTech (Rocky Hill, NJ). Ab to gp100 (clone HMB45) was procured from Lab Vision-NeoMarkers (Fremont, CA). Synthetic gp100 peptides binding to HLA-A2 (209–217 and 209–2M, IMDQVPFSV (27); 280–288, YLEPGPVTA; 457–466, LLDGTATLRL; 154–162, KTWGQYWQV; 476–485, VLYRYGSFSV (28)) and Th peptides binding to HLA-DR7 (74–89, GPTLIGANASFSIALN; 576–590, SLAVVSTQLIMPGQE (29)) were custom-synthesized to >95% purity (PeptidoGenic Research (Livermore, CA) and SynPep (Dublin, CA)).

Development of human mAb specific for MR

Transgenic HuMAb mice, strain HC2/KCo7, with four distinct genetic modifications were used for immunizations (30). These transgenic mice contain a human Ig gene miniloci that encodes unrearranged human H (μ

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[4] Abbreviations used in this paper: pAPC, professional APC; B-LCL, B lymphoblastoid cell; CD40L, CD40 ligand; DC, dendritic cell; β_2m, β_2-microglobulin; Mϕ, macrophage; MR, mannose receptor; sMR, soluble MR.

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and γ) and κ L chain Ig sequences together with targeted mutations that inactivate the endogenous μ- and κ-chain loci. Accordingly, the mice exhibit no expression of mouse IgM or κ, and in response to immunization, the introduced human H and L chain transgenes undergo class switching and somatic mutation to generate high affinity human IgGκ mAbs. Immature human DCs ($1–5 \times 10^6$) were used to immunize mice in CFA. Single-cell suspensions of splenic lymphocytes from immunized animals were fused with the murine myeloma cell line P3X63Ag8.653 (American Type Culture Collection) in the presence of polyethylene glycol. Hybridomas were selected by the addition of HAT 24 h after fusion. Human IgGκ-producing hybridomas were screened by flow cytometry for binding to DCs. Ab specificity was determined by immunoprecipitation and sequencing. Briefly, DCs were lysed using detergent lysing buffer (50 mM Tris-HCl, 150 mM NaCl, 0.02% NaN_3, 100 μg/ml PMSF, 1 μg/ml aprotinin, and 1% Triton X-100). Precleared supernatant was incubated overnight with B11 mAb bound to anti-human IgG-agarose. After washing, the bound proteins were removed from the agarose by boiling in SDS-PAGE sample loading buffer. Samples were applied to precast 4–20% gels (Bio-Rad, Hercules, CA) and separated under nonreducing conditions. The proteins were transferred to a polyvinylidene difluoride membrane and stained with Coomassie Blue. The band corresponding to the B11 Ag was N-terminally microsequenced by Edman N-terminal protein sequencing (Commonwealth Biotechnologies, Richmond VA). The N-terminal 20 aa, LLDTRQFLIYLEDTKRCVDA, were found to share 100% identity with the N-terminal sequence of the human macrophage MR.

Development and purification of fusion construct

A plasmid, pMMV4, encoding the B11 L chain as well as the B11 H chain fused in-frame at its 3′ end to sequences encoding the pmel17 ectodomain was constructed. Briefly, the B11 V_L and V_H sequences were cloned into a mammalian expression vector into which the human Igκ and IgG1 genes had been previously introduced. A multiple cloning site was introduced at the 3′ end of the H chain gene that eliminated the native stop codon and allowed for the in-frame introduction of pmel17 sequences. Pmel17 cDNA was obtained by RT-PCR using RNA extracted from the SK-Mel-3 human melanoma cell line (American Type Culture Collection). The fidelity of all cloning steps was verified by DNA sequencing of the entire B11 H chain fusion and B11 L chain coding regions in pMMV4.

The pMMV4 construct was linearized with *PvuI* and transfected into CHO cells using SuperFect reagent according to the manufacturer's instructions (Qiagen, Valencia, CA). Stable transfectants were selected by maintaining cells in growth medium (αMEM and 10% dialyzed FBS) containing 550 μM G418 (Calbiochem-Novabiochem, San Diego, CA). Subsequently, colonies were isolated using cloning cylinders and subjected to multiple rounds of selection in growth medium containing increasing concentrations of methotrexate (20, 80, and 320 nM). Clonal cell lines that produced the highest amounts of fusion protein were identified by ELISA and selected for further development.

B11-pmel17 fusion protein was purified from cell lysates. Briefly, stable CHO transfectants were harvested, washed with PBS, and resuspended in digitonin lysing buffer (1% digitonin, 0.12% Triton X-100, 20 mM triethanolamine, 150 mM NaCl, 2 mM PMSF, and 10 μg/ml aprotinin, pH 7.8; Sigma-Aldrich, St. Louis, MO) to a concentration of 5×10^7 cells/ml. Cells were incubated on ice for 60 min with frequent vortexing. Cell debris was removed by centrifugation ($16,350 \times g$, 15 min), and the supernatant was collected. The B11-pmel17 fusion protein was purified from the supernatants on a protein A-Sepharose column and subjected to SDS-PAGE under reducing conditions. Proteins were visualized by Coomassie R250 staining or, alternatively, were transferred to nitrocellulose membranes and detected by immunoblotting using alkaline phosphatase-conjugated goat anti-IgG (H&L) (Jackson ImmunoResearch Laboratories, West Grove, PA) in conjunction with the CDP Star chemiluminescent detection system (Roche, Indianapolis, IN) according to the manufacturer's instructions.

Monocyte-derived DC cultures

PBMC (2.5×10^6/ml) were suspended in AIM-V medium (Invitrogen, Carlsbad, CA) supplemented with 5% human AB serum (Biological Specialty Corp.), 40 μg/ml gentamicin, and 50 μM 2-ME (Sigma-Aldrich). PBMCs were allowed to adhere for 90 min at 37°C. Nonadherent cells were gently removed, washed, and cryopreserved. Adherent cells were cultured in the above growth medium supplemented with 25 ng/ml GM-CSF and 100 ng/ml IL-4 (R&D Systems, Minneapolis, MN). Immature DC were harvested on days 5–6 and were either used as stimulators or cryopreserved for later use.

Binding assay

The ability of B11-pmel17 to specifically bind immature DCs was examined in the presence or the absence of either B11 $F(ab')_2$ or soluble recombinant human MR (sMR). $F(ab')_2$ of B11 Ab and an irrelevant human IgG were prepared and purified according to previously published methods (31). A portion of the MR ectodomain was cloned by RT-PCR from RNA prepared from human monocyte-derived DCs (primer pair 5′-GACAAGC TATTTGCGGCCGCGCCATTGAAATTTGAGGG-3′ and 5′-TTCGGT GGGTGGTCGACTCCTTCTGCCCAGTGCTTGCACAC-3′) and expressed as a histidine-tagged fusion protein secreted by stably transfected CHO cells. The sMR was purified from CHO supernatants using Ni-NTA chromatography (Qiagen) according to the manufacturer's suggestions.

For experiments involving $F(ab')_2$, immature DCs were incubated for 1 h on ice with 55 μg/ml of either B11-$F(ab')_2$ or control $F(ab')_2$. B11-Pmel17 was added to a final concentration of 0.3 μg/ml, and samples were incubated at 4°C for an additional hour, then washed with ice-cold PBS. Cell surface-bound B11-pmel17 was detected by the addition of polyclonal rabbit anti-pmel17, followed by incubation with PE-labeled donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories). Binding assays involving sMR were performed as described above,except that B11-pmel17 was preincubated for 1 h at 4°C with purified sMR before incubation with immature monocyte-derived DC. Normal rabbit serum was included as a negative control, and all samples were analyzed by flow cytometry.

Internalization assays

To investigate the mechanism of internalization, DCs were treated on ice with B11-FITC (20 ng/ml) or mannosylated BSA-FITC (400 ng/ml; Sigma-Aldrich) for 30 min in AIMV medium plus 3 μg/ml human γ-globulin (Sigm-Aldrich) and 1% BSA (Sigma-Aldrich) with or without 400 mM sucrose. Cells were then warmed to 37°C for 20 min. After treatment, cells were washed twice with ice-cold PBS containing 1% BSA and 0.05% sodium azide (Fisher Scientific, Fairlawn, NJ) and fixed in ice-cold 1% methanol-free formaldehyde (Polysciences, Warrington, PA) in PBS overnight. Cells were then washed twice with ice-cold PBS containing 1% BSA and 0.05% sodium azide and prepared for imaging. Fixed cells were pelleted and imaged using an MRC-1024 confocal scanning laser microscope system and LaserSharp version 3.2 software (Bio-Rad). 605DF32, 522DF32, and 680DF32 bandpass filters were used for photomultiplier tubes 1, 2, and 3, respectively. Laser power was set at 3% on all lines (488, 568, and 647 nm wavelengths). All cells were imaged using a 63X/1.4NA PlanApo objective with oil and an iris setting of 1.5 for all photomultiplier tubes used in a given experiment. The section thickness is estimated to be at the optimal axial resolution for this system (~0.5 μm). Image acquisition settings included the Kalman filtered mean of three slow scan accumulations with all laser lines. Images were captured as a single section from the center plane of cells and are representative of 30 fields captured/slide.

Generation of B lymphoblastoid cell lines (B-LCL)

Autologous B cell lines were prepared as previously described (32). Briefly, $2.5–3 \times 10^7$/ml PBMCs were infected by incubation with B95.8 supernatants (American Type Culture Collection) for 90 min at 37°C, followed by addition of 5 ml of RP-15 medium (RPMI 1640 supplemented with 20 mM HEPES, 2 mM L-glutamine, 1% sodium pyruvate, 15% FBS, 40 μg/ml gentamicin, and 50 μM 2-ME). Infected B cells were selected for transformation by addition of 10 ng/ml cyclosporin A (Calbiochem-Novabiochem, San Diego, CA) once a week. The original medium was never removed, only diluted with fresh medium. Homogenous B-LCL were obtained within 4–5 wk of culture. Cells were split at this point and expanded initially using a split ratio of 1:2 through higher ratios to 1:5 and 1:10.

T cell stimulation and expansion

Nonadherent PBMC (PBL) were used as a source of T cells from frozen stocks and stimulated with autologous Ag-targeted immature DC every week for 4–5 wk. Briefly, 1.2×10^6/ml DC were exposed to Ag, B11-pmel17 (20 μg) in 1.0 ml of AIM-V serum-free medium for 45 min and allowed to mature with CD40 ligand (CD40L; PeproTech, Rocky Hill, NJ; 20 ng/ml) for 24 h. PBL (2×10^6/ml) were cocultured with Ag-loaded DC in 24-well culture plates (B-D Biosciences, San Jose, CA) in the presence of IL-7 (10 ng/ml; day 0), followed by addition of other cytokines, IL-10 (10 ng/ml) on day 1 and IL-2 (20 U/ml) on day 2. The T cell:DC ratio was maintained at 20 throughout the course of stimulations. IL-2 was added every 3–4 days, whereas restimulations were performed on days 8, 16, and 24 as before, except that the Ag dose was cut by half compared with that used in the preceding stimulation (10.0, 5.0, and 2.5 μg/ml, respectively).

T cells were maintained as bulk cultures (containing both $CD4^+$ and $CD8^+$ T cells) unless otherwise indicated.

Effector T cells (5×10^4 to 1×10^5/flask) were expanded in T25 flasks (Corning Glass, Corning, NY) on allogeneic mitomycin C-treated PBMC feeder layers (2.5×10^6/ml) pooled from three donors with added anti-CD3 Ab (25 ng/ml) and IL-2 at a dose of 20 U/ml. Medium was changed on days 5 and 8 by first removing half the spent medium and replacing with fresh medium containing 50 U/ml IL-2. T cells were harvested and assayed between days 10–12 or were cryopreserved for later use (32).

Cytotoxicity assays

T cells generated with B11-pmel17-stimulated DCs were tested for reactivity against various ^{51}Cr-labeled targets. The targets included autologous B-LCL or TAP-deficient T2 (HLA-A2.1$^+$) and a panel of HLA-matched/mismatched melanoma and nonmelanoma tumor cell lines. Cytotoxicity was determined in a standard 4-h chromium release assay. MHC class I and II restriction was ascertained by assessing the reactivity in the presence of targets preincubated and HLA-specific antisera. All assays were performed in triplicate. Data shown are from a representative experiment of two or three independent experiments performed. The percent specific killing of targets was calculated from the formula: percent specific lysis = [(experimental release$_{cpm}$ − spontaneous release$_{cpm}$)/(maximal release$_{cpm}$ − spontaneous release$_{cpm}$)] × 100. Experimental release is the radioactivity released by CTL in the presence of ^{51}Cr-labeled targets, and spontaneous and maximal release correspond to radioactivity in wells containing no added CTL, i.e., ^{51}Cr-labeled targets in medium and 2% Nonidet P-40 (Igepal CA630; Sigma-Aldrich)-containing medium, respectively. Radioactivity was counted using a gamma counter (Wizard 1470; Wallac, PerkinElmer, Shelton, CT).

Cold target inhibition assay

T cells from donor 1 (HLA-A2$^+$), previously stimulated with DC-B11-pmel17, were tested for reactivity against ^{51}Cr-labeled (hot) SK-Mel 19 melanoma cells (HLA-A2$^+$) in the presence or the absence of unlabeled (cold) HLA-A2$^+$ T2 cells with or without peptides. For inhibition experiments, several gp100 peptides known to bind HLA-A2 were loaded on T2 cells (10 μg/ml for 3.0×10^6/ml cells in AIM-V medium) in the presence of β_2-microglobulin (β_2m; 3.0 μg/ml) for 2 h at room temperature. Peptide-bound T2 cells were washed once in PBS and centrifuged to remove unbound peptides. A 10-fold excess of peptide-loaded or unloaded T2 cells was then added to CTL, followed by addition of labeled SK-Mel 19 targets. The E:T cell ratio was maintained at 40, and the cold:hot target ratio was maintained at 10. T2 cells pulsed with irrelevant HLA-A2 binding peptide (HBVcore$_{18-27}$) served as controls. Percent inhibition of specific lysis = 1 − [(specific lysis in the presence of cold targets)/(specific lysis in the absence of cold targets] × 100.

Proliferation assays

T cells generated from B11-pmel17-treated DCs (5×10^4) were cocultured with autologous DC (5×10^3) not pulsed or pulsed with gp100 peptides (74–89 and 576–590) for 3 days at 37°C in a final volume of 0.2 ml of RP-10 medium. On day 3, cultures were pulsed with [^{3}H]thymidine (1 μCi/well; NEN-PerkinElmer, Boston, MA) for the last 8 h. Cells were then harvested onto filters with a Cell Harvester (Wallac, Shelton, CT) and washed three times with water, followed by a final wash in ethanol. Filters were air-dried and loaded with 20 μl of OptiPhase SuperMix scintillant/well (PerkinElmer, Turku, Finland). Filter-bound radioactivity was counted using a beta scintillation counter (1450 MicroBeta Jet; Wallac PerkinElmer, Downers Grove, IL). MHC restriction was conducted by addition of MHC class I- or II-specific Ab (20 μg/ml) to DC before incubation with T cells.

Results

Characterization of anti-MR mAb, B11

The expression of MR is primarily restricted to tissue macrophages, myeloid DCs, and hepatic endothelial cells (33–36), and as such makes an attractive candidate for targeting Ags to APCs. We generated a human anti-MR mAb, B11, by immunization of human Ig-expressing mice with immature human DCs, followed by standard hybridoma methodology. The specificity of the B11 mAb was investigated by immunoprecipitation experiments with lysates prepared from DCs. Electrophoresis identified a single product band at ~180 kDa (data not shown) with the N-terminal amino acids LLDTRQFLIYLEDTKRCVDA, which share 100% identity with the N-terminal sequence of the human macrophage MR. Interestingly, B11 Ab did not block binding of mannosylated BSA to DCs (data not shown), a property that has been ascribed to other anti-MR Abs (36).

MR-mediated internalization by DCs occurs through macropinocytosis or receptor-mediated endocytosis (37). Macropinocytosis is the predominant mechanism of endocytosis for the MR ligands, dextran, HRP, and mannosylated BSA (22). To determine the mechanism of B11 internalization, experiments were performed under hypertonic conditions that disrupt clathrin-dependent receptor-mediated endocytosis (38). Immature DCs were incubated on ice with or without 400 mM sucrose for 30 min in the presence of either B11 mAb or mannosylated BSA. Cells were then warmed to

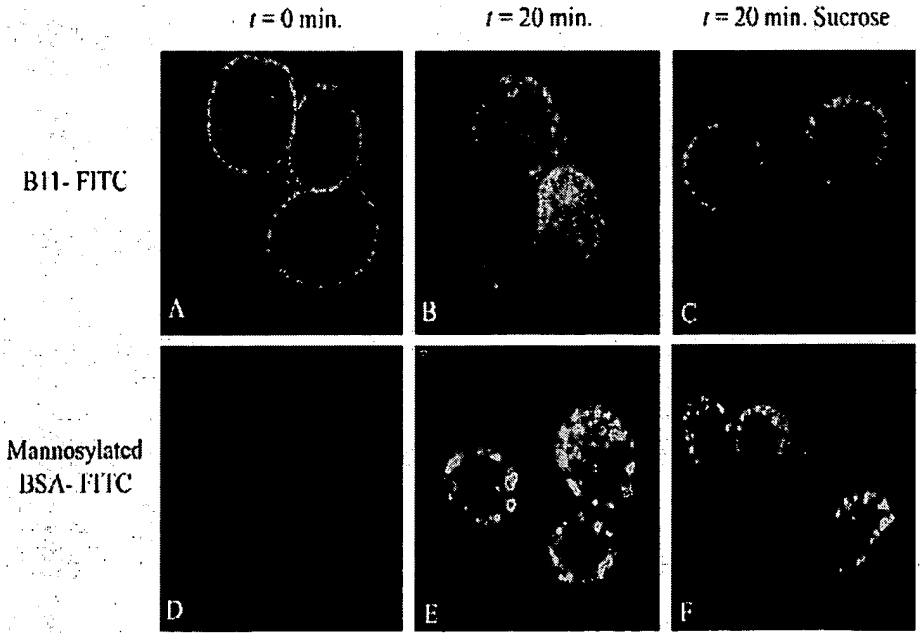

FIGURE 1. Inhibition of clathrin-mediated internalization. Immature DCs were incubated on ice for 30 min in the presence of B11-FITC (*A–C*) or mannosylated BSA-FITC (*D* and *E*). Cells were then warmed to 37°C and incubated for 20 min in the presence (*C* and *F*) or the absence (*B* and *E*) of 400 mM sucrose. Cells were then washed, fixed, and analyzed by confocal microscopy.

37°C and allowed to internalize for 20 min. After being washed and fixed, cells were analyzed by confocal microscopy. When B11 was bound to the MR, its uptake was inhibited by hypertonic shock, indicating that its mechanism of internalization was through clathrin coated-pits (Fig. 1). As expected, the uptake of mannosylated BSA was not inhibited by hypertonic shock, indicating that its mechanism of internalization was not dependent on clathrin coated-pit formation. Even at a 20-fold higher concentration relative to B11, the surface staining by mannosylated BSA-FITC was relatively weak. Subsequent studies revealed that internalized mannosylated BSA-FITC colocalized with nonspecific, fluid phase tracers, whereas vesicles containing internalized B11 excluded the nonspecific tracer (data not shown). In contrast to B11-FITC, the uptake of both mannosylated BSA-FITC and the fluid phase tracer was largely blocked by pretreatment with the phosphatidylinositol 3-kinase inhibitor, wortmannin (data not shown). These results show the rapid uptake of the B11 occurs by a distinct mechanism relative to the uptake of mannosylated BSA.

Biochemical characterization of B11-pmel17

Having demonstrated efficient DC binding and internalization of the B11 mAb, we designed a tumor Ag containing fusion protein for investigation of Ag-specific presentation to T cells. Pmel17 is a melanocyte-specific protein and a splice variant of gp100 that encodes all of gp100 plus an additional seven amino acids (39). The selective expression profile of pmel17/gp100 has made this protein an attractive target for active immunotherapy strategies for the treatment of melanoma (reviewed in Refs. 40 and 41). Despite the fact that only the ectodomain of pmel17 was included in the fusion protein, most B11-pmel17 produced by CHO transfectants remained cell-associated. B11-pmel17 purified from cell lysates was characterized by SDS-PAGE and Western blot analysis (Fig. 2), which revealed a fully assembled Ab fusion protein of the expected size.

Specific binding of B11-pmel17 to immature DCs

The B11-pmel17 fusion protein also retained the functional properties of B11 Ab, as demonstrated by its ability to bind monocyte-derived immature DCs. Furthermore, the pmel17 component of B11-pmel17 does not significantly contribute to its recognition of these cells, because the B11 $F(ab')_2$, which lacks both the Fc and pmel17 regions, is nevertheless able to completely abrogate binding of B11-pmel17 to immature human DCs (Fig. 3). The fact that sMR is also able to abrogate B11-pmel17 binding indicates that B11-pmel17 interacts with MR present on the cell surface of immature DCs.

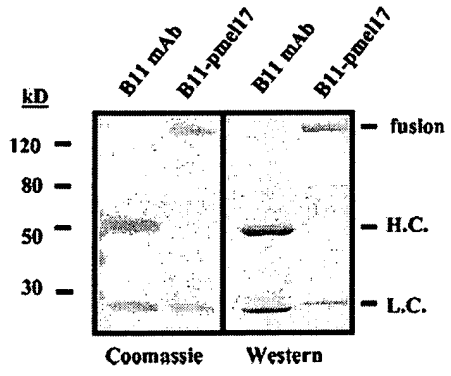

FIGURE 2. Biochemical characterization of B11-pmel. B11-pmel17 fusion product and B11 mAb were purified on protein A columns and analyzed for purity by SDS-PAGE under reducing conditions. Proteins were stained with Coomassie R250 or were transferred to nitrocellulose and visualized with alkaline phosphatase-conjugated goat anti-human IgG (H and L chain specific) in conjunction with a chemiluminescent detection system (fusion, B11 H chain-pmel17 fusion; H.C., B11 H chain; L.C., κ L chain from either B11 or B11-pmel17).

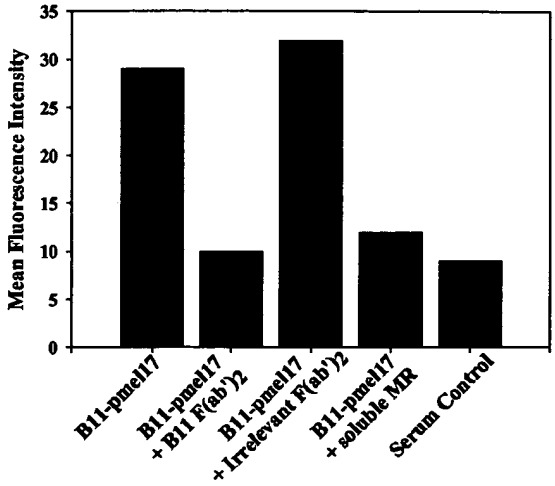

FIGURE 3. B11-pmel17 specifically binds monocyte-derived DC via MR. Binding of B11-pmel17 to immature monocyte-derived DC was examined in the presence and the absence of B11 $F(ab')_2$, an irrelevant $F(ab')_2$, or a purified soluble fragment of the human MR. Normal rabbit serum was included as a negative control. Shown is a representative experiment of three independent experiments performed with similar results.

Induction of an MHC class II-dependent, pmel17-directed Th response

An autologous in vitro culture system incorporating PBL and monocyte-derived DCs was established to investigate the ability of B11-pmel17 to enhance cellular immune responses from two normal donors. Briefly, PBL were stimulated as bulk culture with CD40L-matured, B11-pmel17-loaded DCs. To define the MHC class II-dependent responses, T cells were induced to proliferate in response to specific antigenic stimulation by Ag pmel17-bearing mature DCs. As shown in Fig. 4, T cells were markedly stimulated only in the presence of autologous DCs loaded with sensitizing Ag,

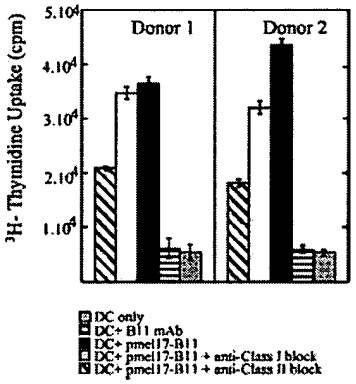

FIGURE 4. Proliferative response to specific stimulation with B11-pmel17 Ag is restricted by HLA class II. T cells from bulk cultures were stimulated with DCs unloaded or loaded with control Ag (B11 mAb, 20 μg/ml) or specific Ag (B11-pmel17, 20 μg/ml) in the presence or the absence of blocking anti-HLA class I (W6/32, 20 μg/ml) or anti-HLA class II (L243, 20 μg/ml) for 3 days. Cultures were pulsed with [^{3}H]thymidine (1.0 μCi/well) for the last 8 h, harvested, and counted. All DC stimulator cells were matured with CD40L (10 ng/ml) before use. Shown is a representative experiment of two independent experiments performed in sextuplicate. Donor 1, $A2^+$ $DR2^+$; donor 2, $A1^+$ $DR7^+$.

B11-pmel17 compared with DC loaded with the vehicle B11 alone or unloaded DC; the specific stimulation was 5- to 6-fold greater than control stimulation. These results also reflect the fact that the response is primarily directed toward the Ag, pmel17, rather than toward the B11 component of the fusion protein. These responses were fairly reproducible in the two donors tested (donor 1, DR2$^+$; donor 2, DR7$^+$). The most significant result pertaining to the HLA class II-dependent, pmel-17-specific response is the observation that anti-HLA-DR-specific Ab (L243), but not anti-HLA-A, -B, or -C-specific Ab (W6/32), was effective in blocking the proliferation, as reflected in reduced uptake of the radioactive tracer.

Synthetic gp100 epitopes can mimic the Th response initiated with pmel17 protein

Taking this analysis a step further, we were curious to determine whether exogenously added helper peptides derived from pmel17/gp100-gp100$_{576-590}$ and gp100$_{74-89}$ would be sufficient to trigger a similar proliferative response using T cells derived from HLA-DR7$^+$ donor. Autologous B-LCL generated from this donor were used, with or without the added peptides, as stimulators. Thus, bulk T cells from DC-pmel17 stimulations were cocultured with synthetic gp100$_{576-590}$ and gp100$_{74-89}$ peptide-loaded B-LCL (at a ratio of 10:1) for 72 h and pulse-chased with tritiated thymidine for the last 8 h. As depicted in Fig. 5, robust proliferative responses were noted for both helper peptides over a range of concentrations tested. These results suggest that the proliferative response is similar in magnitude to that observed with DC-pmel17-induced stimulation (see Fig. 4) and, therefore, is likely to be directed against preprocessed helper peptides being presented by an alternate APC, i.e., a B-LCL. Similar responses were not obtained with T cells derived from the HLA-DR7-negative donor, as expected (data not shown). These results clearly demonstrate that DC targeting of pmel17 tumor Ag using B11-pmel17 fusion protein can result in the stimulation of an HLA class II-dependent Ag directed Th function.

B11-pmel17-treated DCs can direct the development of anti-melanoma cytolytic T cells

A requirement for pmel17/gp100-specific CTL recognition of melanomas is that these tumor cells also process and present pmel17/gp100 peptides in association with matched class I molecules. We examined the ability of pmel7/gp100-specific T cells to recognize a number of cancer cell lines. As shown in Fig. 6, T cells stimulated with DC-B11-pmel17 recognize HLA-compatible SK-Mel

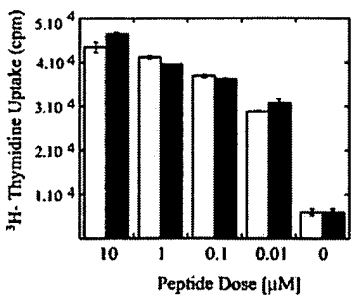

FIGURE 5. B11-pmel17-sensitized T cells proliferate in response to defined Th epitopes derived from gp100. Autologous B-LCLs (donor 2, HLA-DR7$^+$) were loaded with a range of peptide concentrations for 2 h at 26°C, washed, and incubated with CTL for 3 days at 37°C. Cultures were pulsed with tritiated thymidine for the last 8 h and counted. ■, gp100$_{74-89}$ (GPTLIGANASFSIALN); ▨, gp100$_{576-590}$ (SLAVVSTQLIMPGQE) (28). Shown is a representative experiment of two independent experiments performed in sextuplicate.

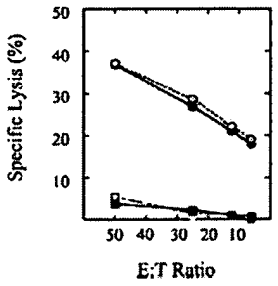

FIGURE 6. Cytolytic effector T cells specific for pmel17 can mount anti-melanoma responses. SK-Mel-31 melanoma cells (targets (T), ●) were pretreated with IFN-γ for 48 h and labeled with ^{51}Cr. Labeled target cells were then added to CTL (effectors (E)) titrated to give different E:T cell ratios, and incubated at 37°C for 4 h. Supernatants were harvested for gamma counting. Autologous B-LCLs served as the HLA-compatible control target (□). For Ab-blocking experiments, SK-Mel 31 cells were preincubated with anti-class I (W6/32; ■) or anti-class II (L243; ○) Ab (20 μg/ml each) at room temperature for 30 min, washed once, and added to CTL. Targets and effector T cells share HLA-A31 (donor 1). Shown is a representative experiment of three independent experiments performed in triplicate with similar results.

31 melanoma targets, but not the completely HLA-matched autologous B-LCL. Importantly, we show that the anti-melanoma response is significantly blocked only in the presence of HLA class I-specific Ab, but not with a class II-specific Ab. To assess the broad specificity of the pmel17-specific CTL, a panel of nine different HLA class I-matched human melanoma targets was tested (Table I). T cells of at least one donor (HLA-A2$^+$) were lytic on a majority of the melanomas tested, albeit to different degrees, whereas T cells from the second donor (HLA-A1$^+$, -DR7$^+$) were not lytic on most targets tested, except one (SK-Mel 31). As the control targets, which included autologous B-LCLs, T24 bladder carcinoma cells, and SK-Br-3, breast carcinoma cells, were not lysed by anti-pmel17 CTL, the T cell response observed was highly specific to an epitope derived from pmel17/gp100 melanoma Ag presented in the context of class I MHC molecules.

Competitive inhibition with peptides reveals multiepitope specificity of CTLs

Previously, several T cell epitopes derived from gp100 have been reported with some currently undergoing clinical evaluation (27, 40, 41). In this regard, the modified synthetic peptide, pmel17/gp100$_{209-217}$ (209–2M; p2T→M) IMDQVPFSV, has been reported to bind HLA-A2 better than the native Ag (ITDQVPFSV). It was therefore interesting to understand whether processing of B11-pmel17 by DCs indeed results in recognition by T cells with specificity for the modified peptide. As depicted in Fig. 7, potent killing was observed with peptide-pulsed B-LCL compared with no peptide B-LCL targets, indicative of an Ag-specific class I-dependent sensitization of CTL.

Using a peptide-pulsed cold target inhibition assay, we have examined four HLA-A2-binding gp100 T cell epitopes (YLEPGPVTA, KTWGQYWQV, VLYRYGSFSV, and LLDGTATLRL) in addition to gp100 209–2M (IMDQVPFSV) for recognition by pmel17-speicific CTL. Thus, only peptides pulsed on cold T2 cells (TAP-deficient), but not T2 alone, were able to inhibit the lysis of labeled HLA-A2$^+$ SK-Mel 19 targets by HLA-A2$^+$ pmel17 CTL (Table II). Furthermore, T2 pulsed with HLA-A2-binding control peptide (HBV core$_{18-27}$: FLPSDCFPSV) was significantly less effective at blocking CTL activity than gp100 peptides. These results demonstrate that DCs are able to process internalized B11-pmel17

Table I. *Induction of a broad anti-melanoma CTL response by stimulation with pmel17 targeted to DC mannose receptor*[a]

Target[b]	HLA Class I Typing A and B Locus	CTL Activity % Specific Lysis[c]	Possible Restriction Element[d]
Melanoma ($gp100^+$)			
Partial HLA class I match			
1. A 375	A1, 2 B44, 57	25.1	A2
2. WM-266-4	A2, 29 B13, 44	20.3	A2, B13
3. SK-MEL 2	A3, 26 B35, 38	36.4	B35
4. SK-MEL 3	A24, − B13, 44	27.7	B13
5. SK-MEL 5	A2, 11 B7, 60	41.9	A2
6. SK-MEL 19	A2, − B51, −	53.6	A2
7. SK-MEL 24	A1, 2 B44, 64	29.8	A2
8. SK-MEL 31	A1, 31 B53, 60	43.7	A31
HLA class I mismatch			
9. SK-MEL 28	A11, − B60, −	11.8	
Nonmelanoma ($gp100^-$)			
10. SK-Br-3 (breast carcinoma)	A3, 11 B18, 40	7.0	
11. T-24 (bladder carcinoma)	A1, 3 B18, 35	6.0	
12. Autologous B-LCL	A2, 31 B13, 35	5.4	

[a] T cells from an $HLA\text{-}A2^+$ donor were stimulated at 8- to 9-day intervals by autologous DC loaded with B11-pmel17. After 4–5 wk, effector T cells were tested for activity in a 4-h chromium release assay.
[b] Targets were pretreated with 100 U/ml IFN-γ for 48 h.
[c] E:T cell ratio = 40.
[d] Restriction element is based on matching HLA alleles of the donor (see B-LCL above) with that of target cells.

fusion protein and present multiple pmel17-derived CD8 T cell epitopes associated with HLA-A2.

Discussion

Evidence has accumulated in recent years to suggest that DCs are central to the generation of T cell responses. They can be customized in vitro and ex vivo to meet different goals of harnessing the immune repertoire. Notwithstanding, however, is the fact that DCs can also capture proteins nonspecifically for eventual presentation to T cells (42). The rationale behind specific targeting of Ags, therefore, appears to be centered on the relative efficiency of Ag processing and presentation in terms of both qualitative (i.e., efficient access to class I pathway) as well as quantitative (i.e., efficient MHC-peptide complex formation) responses (43). Recently, work from Steinman's laboratory (15) has shown that Ab-targeting Ag to another member of the C-type lectin family, DEC-205, results in class I-restricted Ag-specific T cells in vivo. Importantly, their study makes a clear distinction between targeting of Ags in the absence of adjuvant leading to peripheral tolerance and targeting with an adjuvant, shifting the response toward Th1 immunity (44).

In this study we show that selective targeting of Ag and uptake by the MR present on DCs can elicit cellular immunity in a manner consistent with a directed Ag-specific response restricted by HLA class I and II molecules. Confocal microscopy studies have further shown that immature DCs readily internalize B11-pmel17 by receptor-mediated endocytosis, in contrast to the macropinocytic uptake of mannosylated BSA. Interestingly, B11 was found to localize within MHC class I-containing vesicles after uptake, yet no colocalization of B11-pmel17 with MHC class II-enriched compartments has been observed to date (J. E. Connolly, unpublished observations). Bypassing the conventional pathways for Ag processing and presentation has been generally accomplished using synthetic peptides exogenously added to single MHC allele-expressing reporter cell lines (e.g., Tap-deficient T2 cells) or autologous B-LCL, although this is limited to the availability of well-defined HLA-binding peptides. Therefore, in terms of defining an MHC class I or class II-dependent responses beyond Ab-mediated blocking, we have obtained productive T cell responses to synthetic epitopes, previously confirmed by others, although the responses were initiated using the soluble form of the Ag pmel17.

The recognition of HLA-compatible melanoma cell lines by CTL generated to DC-targeted pmel17 Ag represents the ultimate demonstration of an Ag-directed anti-melanoma response. Although HLA-A2 appears to be the major allele contributing to this response, other alleles (-A31, -B13, and -B35) also may be involved in Ag presentation, because the pmel17 CTL also recognizes $pmel17/gp100^+$ targets that lack HLA-A2. Thus, a favorable lytic response appears to be directly related to Ag recognition in the context of a cognate HLA allele present on the targets, as evidenced by lysis of HLA-matched, but not HLA-mismatched, targets. Consistent with this paradigm, SK-Mel-28, although $gp100^+$, is HLA mismatched and therefore ignored as a CTL target. The lytic activity of our CTL lines on multiple targets further suggests that DCs targeted with tumor Ag fused to anti-MR Ab are capable of activating T cells with specificity for multiple Ags associated with diverse HLA-restricting elements contributing to the

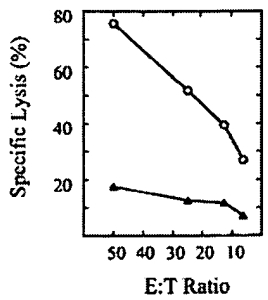

FIGURE 7. Reconstitution of cytolytic activity with peptide-loaded B-LCL. T cells from the $HLA\text{-}A2^+$ donor raised to B11-pmel17 were tested against autologous B-LCLs in the presence (10 μM) or the absence of 209–2M peptide. B-LCLs were loaded with peptide at 26°C for 16 h, washed, and labeled with ^{51}Cr for 90 min. Labeled B-LCLs loaded (O) or unloaded (▲) with peptide were then added to CTL, and the assay was performed as described in Fig. 3. Shown is a representative experiment of two independent experiments performed in triplicate.

Table II. *Competitive inhibition of SK-Mel 19 lysis by gp100 peptide-pulsed cold targets reveals multiepitope presentation by B11-pmel17-targeted DC*[a]

Cold Targets Added	Inhibition of Specific Lysis (%) ± SD
None[b]	
T2 only	15 ± 0.55
gp100 peptides	
T2 + YLEPGPVTA	56 ± 2.90
T2 + IMDQVPFSV (209-2M)	70 ± 1.22
T2 + KTWGQYWQV	69 ± 1.88
T2 + VLYRYGSFSV	84 ± 0.66
T2 + LLDGTATLRL	82 ± 1.66
Control peptide	
T2 + FLPSDCFPSV[c]	28 ± 9.50

[a] Cold target inhibition of SK-Mel 19 lysis. T cells from normal HLA-A2+ donor previously sensitized with autologous DC-loaded B11-pmel17 were tested for lytic activity against HLA-A2$^+$ ^{51}Cr-labeled (hot) SK-Mel 19 targets (E:T cell ratio = 40) in the presence or the absence of unlabeled (cold) targets (TAP-deficient HLA-A2$^+$ T2 cells) pulsed with HLA-A2-restricted gp100 synthetic peptides (10 μM) and β_2m (3.0 μg/ml). Cold:hot target ratio = 10.

[b] Specific lysis of SK-Mel 19 target cells in the absence of cold targets was 27%.

[c] Peptide modified at position 6; Y→C from the parental sequence of $HBVc_{18-27}$: FLPSDYFPSV.

effector response. This approach, if applied, could be particularly advantageous to target HLA class I Ag loss variants that would otherwise contribute to tumor escape mechanisms, an undesirable fallout due to selection pressure induced by vaccination with single immunodominant epitopes.

Finally, our results demonstrate that targeted delivery of whole proteins to DC via MR can contribute to the development of productive antitumor T cell responses that could be readily adapted to potentially any HLA system for studying T cell responses to Ags in cancer and infectious disease.

Acknowledgments

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